

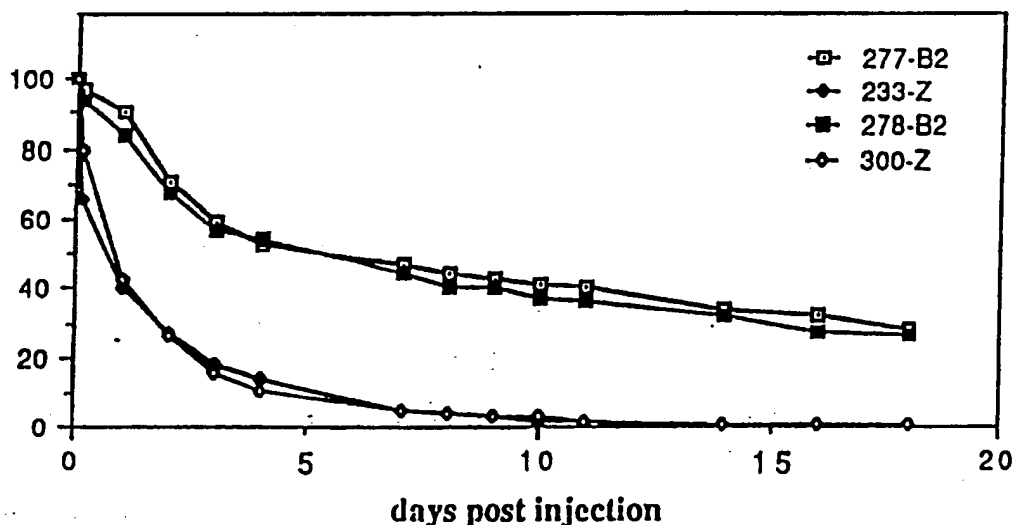


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/02, 47/48, C07K 13/00 C12N 15/62		A1	(11) International Publication Number: WO 91/01743
			(43) International Publication Date: 21 February 1991 (21.02.91)
(21) International Application Number: PCT/SE90/00509		(74) Agent: AWAPATENT AB; Box 7645, S-103 94 Stockholm (SE).	
(22) International Filing Date: 31 July 1990 (31.07.90)			
(30) Priority data: 8902638-9 1 August 1989 (01.08.89) SE		(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.	
(71) Applicant (for all designated States except US): CEMU BIO-TEKNIK AB [SE/SE]; Kungsgatan 46, S-743 21 Uppsala (SE).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only) : NYGREN, Per-Åke [SE/SE]; Pilotgatan 22, S-122 51 Enskede (SE). WIG-ZELL, Hans [SE/SE]; Oscar Bäckströmsvägen 11, S-126 54 Hägersten (SE). UHLÉN, Mathias [SE/SE]; Kvarnbogatan 30, S-752 39 Uppsala (SE).			

(54) Title: STABILIZED PROTEIN OR PEPTIDE CONJUGATES

% remaining label in plasma



(57) Abstract

A process for extending the half-life *in vivo* by a biologically active protein or peptide, characterized by covalently coupling said protein or peptide to a polypeptide fragment capable of binding to a serum protein, whereby when administering the resulting protein or peptide conjugate its binding to the serum protein results in extended biological activity; the use of the protein or peptide conjugate above for manufacturing a medicament which, when administered to a mammal including man, shows extended half-life *in vivo*; and a method of therapeutic or prophylactic treatment.

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark			US	United States of America

Stabilized protein or peptide conjugates.

The present invention relates to the use of biologically active proteins or peptides in the form of products showing improved stability in vivo, i.e. extended half-life, and the invention relates inter alia to the use of recombinant vectors capable of replication in a host cell to produce such useful products. More specifically, the invention relates to a protein or peptide conjugate capable of selective binding to a host protein or macromolecule, thus stabilizing the biologically active protein or peptide in this host. The invention also extends to a process for extending the half life in vivo of a biologically active protein or peptide and to the use of a protein or peptide conjugate resulting from such process for the manufacture of a medicament.

Although the present invention in the following will be mainly illustrated through protein or peptide conjugates produced by recombinant DNA technology the invention is not limited to such production system but is equally useful when such protein or peptide conjugate is prepared by chemical covalent coupling of its constituents.

Gene fusion is a procedure wherein the coding sequence of two or more genes are spliced together to form a combined gene which on expression in a suitable host organism will produce a fusion product wherein the separate proteins or polypeptides coded for by the respective genes are fused together into a single molecule.

The rapid depletion of bioactive proteins in vivo is, in some cases, a limiting factor for the efficiency of therapeutic compounds. Examples of products of potential clinical interest with short half-lives in vivo are components such as soluble human CD4, having an interest in the treatment of AIDS, which have a half-life in rabbit of 15 minutes (Watanabe et al., Nature, 337, 1984, 267-270) and human t-PA used in the treatment of blood clots with a halflife of only 2-3 minutes in humans (Hollander, Critical Reviews in Biotechn. 6, 1987, 253-271). Such short half-lives of therapeutically

interesting proteins might make it necessary to distribute the compound to the patient either with a high initial dose or with many repeated distributions in order to keep the level of the compound at a clinically relevant level. This reduces the cost effectiveness of the drug and might cause negative side-effects due to the high doses necessary.

To overcome these problems several systems for slow release of drugs have been designed, in which the therapeutic is encapsulated by physical means to release the drug in a delayed manner (i.e. entero or depot tablets) or is delivered as a pro drug, inactive until chemically modified within the patient. In this way, it is in some cases possible to prolong the action of the therapeutic, although the actual in vivo half-life of the compound in circulation has not increased.

Recently, an alternative strategy has been described using fusions between a recombinant protein and a host protein such as IgG (Capon et al., Nature 337, 1989, 525-531) or IgM (Karjalainen et al., Nature 339 (1989) 68-70). In this way, the half-life of recombinant soluble CD4 in vivo was shown to be substantially prolonged. However, this strategy of distributing therapeutically interesting compounds have the disadvantage that unwanted immunological reactions are possible and that the half-life of the thus produced recombinant fusion protein might not be substantially prolonged.

Also for the development of vaccines and other immunostimulatory preparations, a rapid depletion of the antigen from the circulation might decrease the immune response. In order to present the antigen to the immune system in an efficient manner different vehicles have been developed, increasing the immune response. (Allison et al. Journ. of Imm. Methods, 95 (1986), 157-168).

This strategy is often accompanied by simultaneous injection with weakened or killed pathogens such as in Freund's complete adjuvant (FCA). However, these formula have the potential risk of being toxic to the receiver and

might lead to denaturation of the protein, thus limiting the use of this strategy for the distribution of therapeutical proteins.

The present invention provides new improved means to facilitate the stabilization of proteins and polypeptide products in vivo. According to the present invention this is achieved by coupling, such as by fusion of the desired biologically active protein or polypeptide to a binding-protein capable of selective binding to a host protein or macromolecule thus stabilizing the desired protein in said host. By selective binding to a patient protein with a relatively long half-life, the depletion of the fusion protein is retarded. By the term "patient" used in the present disclosure is intended a living animal, especially a mammal including man.

In accordance with a preferred aspect of the present invention gene fusion is used to combine a first DNA-sequence coding for a bindingprotein with a second DNA-sequence coding for a desired protein or polypeptide into a functional gene capable of expressing the fusion product of said desired binding-protein part.

Due to the binding-ability the produced protein or polypeptide is stabilized in vivo in the receptor host.

Accordingly, the present invention is based on the surprising finding that the half-life in vivo of a biologically active protein or peptide can be substantially prolonged by covalently coupling such protein or peptide to a polypeptide fragment capable of binding to a serum protein. This finding was totally unexpected and could not be predicted from available scientific knowledge.

Thus, according to one aspect of the invention, there is provided a process for extending the half-life in vivo of a biologically active protein or peptide, such process comprising the steps of covalently coupling the protein or peptide to a polypeptide fragment which is capable of binding to a serum protein. When administering the protein or peptide conjugate resulting from such process the binding thereof to

the serum protein results in substantially extended biological activity due to increased half-life thereof.

According to a preferred embodiment of this aspect of the invention said polypeptide fragment is capable of binding to serum albumin, such as a serum albumin of mammal origin, for example human serum albumin.

The binding polypeptide fragment of the conjugate preferably originates from staphylococcal protein G.

Another aspect of the invention is constituted by the use of the protein or peptide conjugate as defined above for the manufacture of a drug or medicament which, when administered to a mammal including man, shows extended halflife in vivo thus prolonging the biological activity of the conjugate.

A preferred aspect of the present invention is thus the provision of a recombinant DNA cloning vehicle or vector comprising a DNA sequence coding for a desired protein or polypeptide operatively linked to a DNA sequence coding for binding mediating part, such that said DNA-sequences together code for a fusion protein of said desired protein or polypeptide, said binding mediating part being capable of selectively binding to a protein or macromolecule present in the patient to be treated..

By transforming a compatible host organism with said vector to permit expression of the above combined DNA sequence and culturing the host organism in a nutrient medium the corresponding binding mediating fusion protein or polypeptide will be produced. Host cells producing functional fusion proteins should be used, which could be bacterial cells, such as *Escherichia* or eukaryotic cells, such as fungi, insect cells, plant or mammalian cell cultures. The transformation of the hosts may be effected with well-known methods.

Said fusion protein of said desired protein or polypeptide and said binding mediating protein produced by the cultured host organism can be efficiently isolated from the cell culture by means of standard protein purification me-

thods such as size exclusion chromatography, ion exchange chromatography or affinity purification using a suitable ligand immobilized to a suitable carrier.

5 If the fusion product is secreted into the surrounding medium the purification may be initiated directly from the medium. If, on the other hand, the fusion product remains within the cells the latter have to be ruptured before such purification can be effected. Rupture of the cell walls may be effected in conventional manner by, e.g., high pressure, 10 ultrasonication, homogenization, shaking with glass-beads etc. In cases where the product is trapped within the periplasmic space between two cell membranes, as in gramnegative bacteria, an osmotic shock procedure may be used to release the product into the suspension medium. Any other treatment 15 of the cultured cells or the growth medium prior to the isolation of the fusion product is, of course, also within the scope of the invention.

In conventional manner the said fusion protein in solution is injected in vivo into the recipient. Due to the 20 part mediating binding to a patient protein or macromolecule the stability of the desired protein or polypeptide is increased.

Alternatively, formation of complexes between said fusion protein and the appropriate patient protein or macromolecule can be accomplished in vitro, whereafter the said 25 complexes are injected into the recipient.

The methods for preparing solution of said fusion protein for injection are well-known and need not be described in any detail herein.

30 The conditions suitable for in vitro complex formation should, of course, be chosen with regard to the particular binding mediating protein and desired protein or polypeptide involved.

35 An example of such part mediating specific binding to a patient protein is the albumin binding regions of streptococcal protein G. (Nygren et al., Journ. of Mol. Recogn. 1,

(1988), 69-74). Serum albumin with a half-life in humans of 19 days is the most abundant protein in the serum (40g/l) and one of its functions is to bind molecules such as lipids and bilirubin. (T. Peters Jr., Advances in Protein Chemistry, 37 (1985) 161-245).

As the albumin-binding regions of streptococcal protein G, designated A1B1A2B2A3, or parts thereof, have a highly specific binding to serum albumin (Nygren et al., Journ. of Molec. Recogn. 1 (1988) 69-74) it is conceived that this protein could be used to construct recombinant fusion proteins, which bind to serum albumin and are carried around in the patient with a distribution resembling serum albumin.

Other examples of parts mediating specific binding to host proteins or macromolecules are receptors, such as the IgG-binding regions of staphylococcal protein A (Uhlén et al., J. Biol. Chem. 259, 1695-1702 (1984)) or streptococcal protein G (Guss et al. EMBO.J. 5, 1567-1575 (1986)) or the staphylococcal fibronectin receptor (Kuusela R., Nature 276, 718-720 (1978)).

One valuable use of such a fusion product is when the protein fused to the part mediating binding to patient proteins or macromolecules has a therapeutic function. In such cases a prolonged in vivo half-life of the desired protein or polypeptide is essential for its clinical use. Examples of such therapeutic proteins or polypeptides are soluble CD4-receptors for AIDS/HIV-treatment, tissue plasminogen activator (tPA) for dissolving blood clots present in the recipient injected and hormones used for growth stimulation (hGH, IGF-I, IGF-II, TNF, EGF) or any other clinically relevant function (i.e. insulin, relaxin).

Another valuable use of the invention is for the production of monoclonal and polyclonal antibodies.

According to the invention a recombinant protein, to which one wants to obtain antibodies, is fused to a binding protein to prolong the half-life in circulation in vivo of said recombinant protein. The longer half-life provides a

longer exposure to the immune system and thus will give higher titers than conventional methods.

Yet another valuable use of the invention is in the production of vaccines. Recombinant proteins used in vaccines can thus be stabilized in vivo, which can make adjuvants superfluous and in general give higher immunological response.

As appears from the above a crucial part of the present invention is the provision of the recombinant DNA structure or vector comprising the combined gene coding for the present fusion protein or polypeptide and capable of transforming a host cell to permit expression thereof and production of the fusion product. The present invention is intended to encompass any such vector irrespective of how it has been obtained using, for example, various restriction enzyme cutting, ligating, transforming and screening techniques well-known in the art as well as any appropriate vector materials and host-organisms. Thus, the DNA sequence coding for the desired protein or polypeptide may be inserted into a suitable vector and the binding coding DNA sequence inserted subsequently, or vice versa: or the two DNA sequences may be introduced simultaneously into the vector. It is also possible to insert the respective DNA sequences in parts thereof into the vector. Further the two DNA sequences may be arranged with either the binding coding sequence or the sequence coding for the desired protein or polypeptide at the 5'-end or start of the combined gene. The special techniques for accomplishing such insertions and combinations with maintained correct reading frames, including the provision of suitable restriction sites therefore, are well-known per se in the art.

The invention also covers a recombinant DNA molecule comprising the recombinant DNA sequence as described above and fused 3' thereof at DNA level a production gene. By this arrangement such molecule obtains the ability to express a fused protein in a suitable host.

Finally, the invention covers a plasmid vector compri-

using the recombinant DNA molecule as described above. The invention also extends to bacterial or eukaryotic cells harbouring the recombinant DNA molecule defined above. The molecule can be inserted in the chromosome of the cell but may also be contained in a autonomously replicating vector, such as plasmid, phage or virus.

The invention will in the following be further illustrated by non-limiting examples with reference to the appended drawings wherein:

Fig. 1 is a schematic drawing of the streptococcal protein G gene (as described by Olsson et al. in Eur.J. of Biochem. 168, pp 318-324) and the constructs containing fragments thereof. For comparison is also shown the construct encoding the Z protein. In row A: pEZT; row B: pB2T; row C: the protein G gene and in row D: pBBCD4;

Fig. 2 shows the levels of label remaining in the blood circulation during an 18-day period in Macaque monkeys injected with ^{125}I -labelled proteins B2 and Z. Values are relative to levels observed 20 minutes post injection;

Fig. 3 shows in lane 1 and 2 an analysis by SDS-PAGE of HSA-affinity purified proteins from the culture medium of E.coli cells harboring pBBCD4 (material in lane 2 is diluted 10 times relative to lane 1.) Lane M: marker proteins with molecular weights as indicated.

Fig. 4 is a schematic drawing of the proteins encoded by the different plasmid constructs used in the example: row A: pB1B2T, row B: pBB-CD4-BB, row C: the complete extracellular part of the human CD4 receptor (KabiGen AB, Stockholm, Sweden);

Fig. 5 shows the result from the modified radioimmunoassay for the analysis of the biological activity for the BB-CD4-BB fusion protein; and

Fig. 6 shows the levels of label remaining in the blood circulation during a 48 hour period in mice injected with ^{125}I -labelled proteins BB, BB-CD4-BB and CD4. Values are relative to levels observed 20 minutes after injection.

Starting materials

E.coli strain RRIΔM15 (Langley et al. Proc.Natl.Acad. of Sci., USA, 72, 1254-1257 (1975)) was used in the examples.
5 The cloning vehicles used were:

pEZZT308 (Nygren et al., J.of Molec.Recogn. 1, 69-74 (1988))

PEG (Eliasson et al., J.of Biol.Chem. 263, 4323-4327 (1988))

10 pUC418 (kindly provided by Dan R. Littman at University of California, San Francisco).

pB1B2 (Nygren et al., J. of Molec.Recogn. 1, 69-74 (1988)).

15 All the strains and vectors are available at the Dept. of Biochemistry, Royal Institute of Technology, Stockholm, Sweden.

Plasmid pNP-3 has been deposited on June 14, 1989 at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany, and given the accession number DSM
20 5394, in accordance with the Budapest Treaty.

Oligonucleotides:

NYPE-1: 5'-CGAATTCGCCTAACGGTATGCAGGGAAACAAAGTGGTGCTGGGC-3'

NYPE-2: 5'-CGGATCCAGGCATCACGATGTCTATTTGAACTCGAGC-3'

were custom made by KabiGen AB using solid phase technology.

25 PCR reactions were carried out on a Techne programmable Dri-Block PCH-1.

Buffers and Media

TSB: Tryptic Soy Broth, made up to one litre and autoclaved.

30 TST: TRIS/HCl (25mM) pH 7.4, 150 mM NaCl, 0.05% Tween 80.

Osmotic Shock solution I: 20% sucrose, 0.3 M TRIS/HCl pH 8.1, 1mM EDTA.

Osmotic Shock solution II: 5mM MgCl₂ (0°C).

35 SDS-PAGE loading buffer: 2.5% SDS (sodium dodecyl sulphate), 5% dithiothreitol (DTT),

0.01% Bromophenol blue.
10x PCR-buffer: 10mM TRIS/HCl, pH 8.3, 5mM KCl,
1.5 mM MgCl₂, 0.01% gelatin
PBS: 0.05 M Sodium phosphate pH 7.1,
0.9% NaCl.

PCR-amplification

An amplification mixture was prepared consisting of the template pUC418 (8 ng/μl), oligonucleotides NYPE-1 and NYPE-2 (each 0.2 μM), 1x PCR-buffer, dNTP's (each 0.2 mM) and 2 units of Taq-polymerase (Stratagene). The time/temp profile used was 92°C (1 min), 50°C (2 min) and 72°C (1 min). This cycle was repeated 35 times.

Protein labelling

After lyophilization proteins were resoluted in distilled water to a concentration of 4mg/ml. 100 μg (25 μl) protein, 50 μl of 0.2 M Na-phosphate buffer (pH7.2), 50 μl Enzymobeads (BioRad Inc.) and 25 μl of 1% β-glucose was mixed with 1 mCi Na¹²⁵I (10 μl) and incubated for 20 min. The supernatant was subsequently loaded on a 5 ml G-25 Superfine Sephadex column (Pharmacia, Sweden) previously equilibrated with PBS (0.1% gelatin). Elution with the same buffer and collection in small fractions efficiently separated labelled proteins from free Na¹²⁵I.

Affinity purification of proteins

Cells harboring the different constructs were grown overnight in Tryptic Soy Broth (TSB) supplemented with Ampicillin 70 mg/l. After centrifugation at 5000 g, the periplasmic content was released using an osmotic shock procedure according to Nossal and Heppel (J. of Biol.Chem. 244, 3049-3062) involving incubation first with 20% sucrose, 0.3 M TRIS/HCl pH 8.0, 1mM EDTA followed by 0.5 mM MgCl₂ (0°C). Shock lysates were loaded directly on IgG-Sepharose (Z) or HSA-Sepharose (B2) respectively. After washing with 1xTST (25

mM TRIS/HCl pH 7.4, 0.15 M NaCl, 0.05% Tween[®] 80) followed by 0.5 mM NH₄Ac, pH 6.0, proteins were eluted with 0.5 M HAc, pH 2.8. The absorbtion at 280 nm was measured and relevant fractions were lyophilized.

5

Distribution of proteins in Maquques

Four Maquques in the range of 6-7 kg were injected with approximately 100 µg of labelled protein using a leg vein. At each sample collection 0.5 ml blood was withdrawn for further analysis. For all samples taken during the 18-day period, the actual measurement of radioactivity was performed on day 18, to eliminate errors due to the half-life of the isotope.

10

Fractionated ammonium sulphate precipitation

Precipitation with ammonium sulphate was performed using standard techniques at 40 and 70% of saturation on 150 µl plasma collected 24 hours after injection.

15

Routine Methods

Methods used routinely in molecular biology are not described (like the use of commercial restriction enzymes, DNA-ligations, Bal 31 exonuclease, S1 nuclease and Klenow polymerase, transformation of E.coli and isolation of plasmid DNA).

20

In order to analyze protein fractions by SDS-PAGE using the PHAST-system (Pharmacia, Uppsala, Sweden), the samples were dissolved in loading buffer (2.5% SDS, 5% Dithiothreitol (DTT) and 0.01% Bromphenol blue). Gradient (8-25% polyacrylamide gels with 5% SDS were run at 10mA for 30 min and subsequently stained with Coomassie-blue.

25

EXAMPLE 1

Plasmid pEZZT308 (Nygren et al., Journ.of Mol.Recogn. 1, 69-74 (1988)), encoding a synthetic divalent IgG-binding domain, ZZ, preceded by the transcription, translation and

30

secretion signals of staphylococcal protein A (SPA), was digested with the restriction endonuclease BglIII, thus releasing a 174 basepair fragment. After recovery from an agarose gel the vector part was religated to yield pEZT, encoding a single IgG-binding domain Z (Fig. 1).

Eliasson et al. (Journ.of Biol.Chem. 263, 4323-4327 (1988)) have described the construction of the plasmid pEG, encoding a protein consisting of the B2, A3, C1, D1 and C3-regions of streptococcal protein G, mediating binding to both IgG and HSA. In order to subclone a fragment encoding only a HSA-binding protein, plasmid pEG was digested with restriction endonucleases Not I and Pst I, releasing a 640 basepair fragment. This was ligated to the purified vector fragment of pEZZT308, previously digested with the same endonucleases. The resulting plasmid pB2T (Fig. 1) encodes a HSA binding protein designated B2 under the same control signals of SPA as above.

Over night cultures of E.coli cells harboring the plasmid pEZT or pB2T were harvested using an osmotic shock procedure. The lysates were loaded directly on columns of IgG-(Z) or HSA-Sepharose (B2) for affinity chromatography. After lyophilization the purified proteins were resoluted and ¹²⁵I-labelled.

In total four Macaque monkeys were intravenously injected with labelled proteins. Individuals # 300 and 233 were given Z-protein and individuals #277 and 278 B-protein. Twentyfour hours after injection the distribution of labelled protein within the plasma was analyzed by fractionated ammonium sulphate precipitation at 40% and 70% of saturation, respectively.

TABLE 1

Individ- dual	Protein	cpm.start	cpm.pellet	cpm.pellet
			40%	70%
300	Z	14833	11733(79%)	440(3.0%)
233	Z	8615	6069(70%)	426(4.9%)
277	B	17197	260(1.5%)	14273(83%)
278	B	22170	346(1.6%)	18242(82%)

As shown in Table 1, in serum originating from apes injected with Z-protein, the majority of label was found in the precipitate at 40% of saturation fraction. This founding was expected as at this level of saturation the precipitate mainly consists of the immunoglobulin content of the serum.

In contrast, in plasma from monkeys injected with B2-protein, the label was found to be located in the precipitate at 70% of saturation. At this level of saturation the precipitate mainly consists of serum albumin. These two results indicate that both recombinant proteins behave as expected in vivo as regarded to their respective affinity.

Furthermore the clearance of the two proteins in the apes was followed during an 18-day period. Twenty minutes after injection the amount of label present in the blood was determined as a reference value. The levels of label remaining in the blood during the period was compared to this start value.

As can be seen in Fig. 2, the levels of label in both apes injected with Z-protein is rapidly decreasing, approaching 10% already after 6 days. This effective clearance might in part be explained by immunological responses to complexes formed between Z-protein and IgG.

Interestingly, in apes injected with B2-protein, the levels of labelled protein present in the blood remains high during the entire 18-days period. After an initial decrease, probably due to a distribution of B2/HSA complexes to the extravascular albumin pool, the levels remaining in both apes resembles the expected declination as regarded to the turn-

over of an average HSA-molecule with a half-life in humans of 19 days. (T. Peters Jr., Advances in Protein Chemistry, 37, 161-245 (1985)).

5 EXAMPLE 2

Plasmid pB1B2 was digested with restriction enzymes EcoRI and Sall, treated with Klenow polymerase and religated to yield pB1B2 Δ R/S. A synthetic oligonucleotide (5'-TGCAAGATCTTTCAATTTCCCTATCCTCGAGAATTCTAAGCTT-3' and its
10 complementary sequence) was inserted in pB1B2 Δ R/S previously cleaved with PstI and HindIII, giving rise to plasmid pB1B2HIV resistant to PstI. A multipurpose cloning linker derived from M13mp18 was cloned between the EcoRI and HindIII restriction sites, resulting in expression of the LacZ' gene
15 positioned immediately downstream. The resulting plasmid was designated pB1B2HIVmp18.

A region encoding aminoacids 1-177 of the mature human CD4 T-cell receptor was in vitro amplified from plasmid pUC418 using the oligonucleotides NYPE-1 and NYPE-2 as pri-
20 mers for the polymerase chain reaction (PCR). After digestion with restriction enzymes EcoRI and BamHI the fragment was ligated into the multilinker of pB1B2HIVmp18 encoding the serum albumin binding domains of streptococcal protein G. The resulting plasmid designated pNP-3 thus encodes a fusion pro-
25 tein consisting of said serum albumin binding region and domains E1 and E2 of the human CD4 molecule involved in the binding to glycoprotein gp120 of HIV-I (Bedinger et al., Nature 334 (1988) 162-164).

E.coli RR1 Δ M15 cells harboring plasmid pNP-3 were cul-
30 tivated at 30°C over night. Analysis on SDS-PAGE on proteins from culture medium affinity purified on HSA-Sepharose shows that the fusion protein is stable in the host and has an apparent molecular weight of 48 kDa (Fig. 3) which is in accordance with estimations from the deduced amino acid sequence.

EXAMPLE 3

Plasmid pB1B2 was digested with endonucleases EcoRI and HindIII to release a 650 bp fragment encoding the serum albumin binding BB domains of streptococcal protein G. This
5 gene fragment was inserted into plasmid pNP-3 previously digested with EcoRI and HindIII. The resulting construction (Fig. 4) designated pEBB-CD4-BB thus encodes a tripartite fusion protein where the CD4 part is flanked by two serum albumin binding regions. E.coli RR1 M15 cells harboring the
10 pEBB-CD4-BB plasmid were grown overnight at 30°C in TSB (Tryptic Soy Broth) containing Ampicillin (100 mg/l). BB-CD4-BB protein was affinity purified from the culture medium using HSA-Sepharose according to standard procedures. Reference CD4 protein containing the complete extracellular region of the human CD4 receptor was obtained from KabiGen
15 (Stockholm, Sweden).

Culture media from an overnight culture of E.coli RR1 M15 cells harboring the plasmid pEBB-CD4-BB was passed through an HSA-Sepharose column. Eluted proteins were analyzed by SDS-PAGE and a major band was seen, with an apparent
20 Mr of 73.000, as expected from the deduced amino acid sequence.

Lyophilized BB-CD4-BB protein was dissolved in PBS-buffer and analyzed for gp120 binding activity in a modified competitive radio immunoassay.
25

Microtiter plates were coated with mouse monoclonal antibodies (F58/H43, P.A. Broliden et al., 1990, J. of Virology, 54, 936-940) for an HIV gp120 determinant using standard procedures. After washing with PBST-buffer the wells
30 were incubated with gp120 protein in PBS-buffer (L. Lasky et al., 1986, Science 233, 209-212). After rinsing with PBST-buffer, BB-CD4-BB at different concentrations was allowed to compete with labelled CD4-protein (KabiGen AB, Stockholm, Sweden) in binding to the immobilized gp120
35 protein. After washing the cpm in the wells was determined using standard methods. As negative control BB protein was

used obtained from cultivation of E.coli cells harboring plasmid pB1B2T encoding the BB domain of streptococcal protein G followed by the trpT termination signals (J.Mol.Recognition (1988), 1 (69-74).

5 As shown in Fig. 5 the characteristics of the inhibition obtained for increasing amounts of BB-CD4-BB is significantly different from the control, indicating a true biologic activity.

10 In order to investigate serum half lifes Balb/C mice were injected using a tail vein with labelled proteins BB, BB-CD4-BB and CD4, respectively. At different time points during a 48 hour period blood samples were taken and the cpm per mg plasma determined.

15 As reference (100%) value, the cpm per mg at 20 min post injection was used.

 The results shown in Fig. 6 indicate that the strategy to fuse the CD4 molecule to the serum albumin receptor results in an increased serum half-life for this hybrid molecule (BB-CD4-BB) as compared to the unfused counterpart (CD4).

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 9, line 17-20 of the description ¹**A. IDENTIFICATION OF DEPOSIT ²**Further deposits are identified on an additional sheet ☐ ³Name of depositary institution ⁴DSM DEUTSCHE SAMMLUNG VON
MIKROORGANISMEN UND ZELLKULTUREN GmbHAddress of depositary institution (including postal code and country) ⁴Mascheroder Weg 1 B
D-3300 BRAUNSCHWEIG, DEDate of deposit ⁵

12 June 1989

Accession Number ⁶

DSM 5394

B. ADDITIONAL INDICATIONS ⁷ (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ⁸** (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS ⁹** (leave blank if not applicable)The indications listed below will be submitted to the International Bureau later ⁹ (Specify the general nature of the indications e.g., "Accession Number of Deposit")

DSM 5394

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)Inger Willén
(Authorized Officer) Inger Willén☐ The date of receipt (from the applicant) by the International Bureau ¹⁰

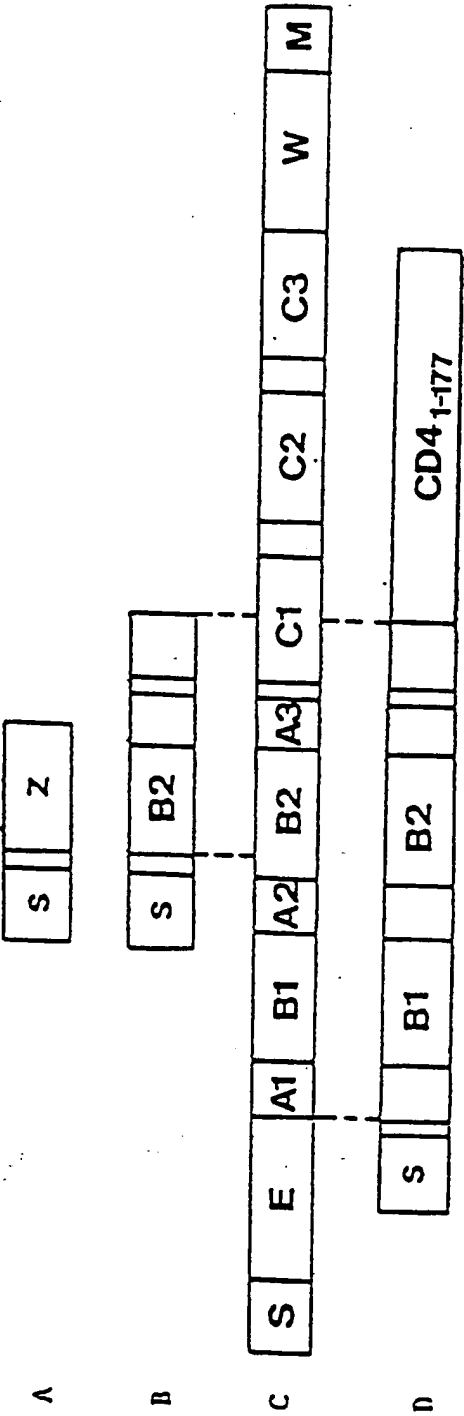
was

(Authorized Officer)

CLAIMS

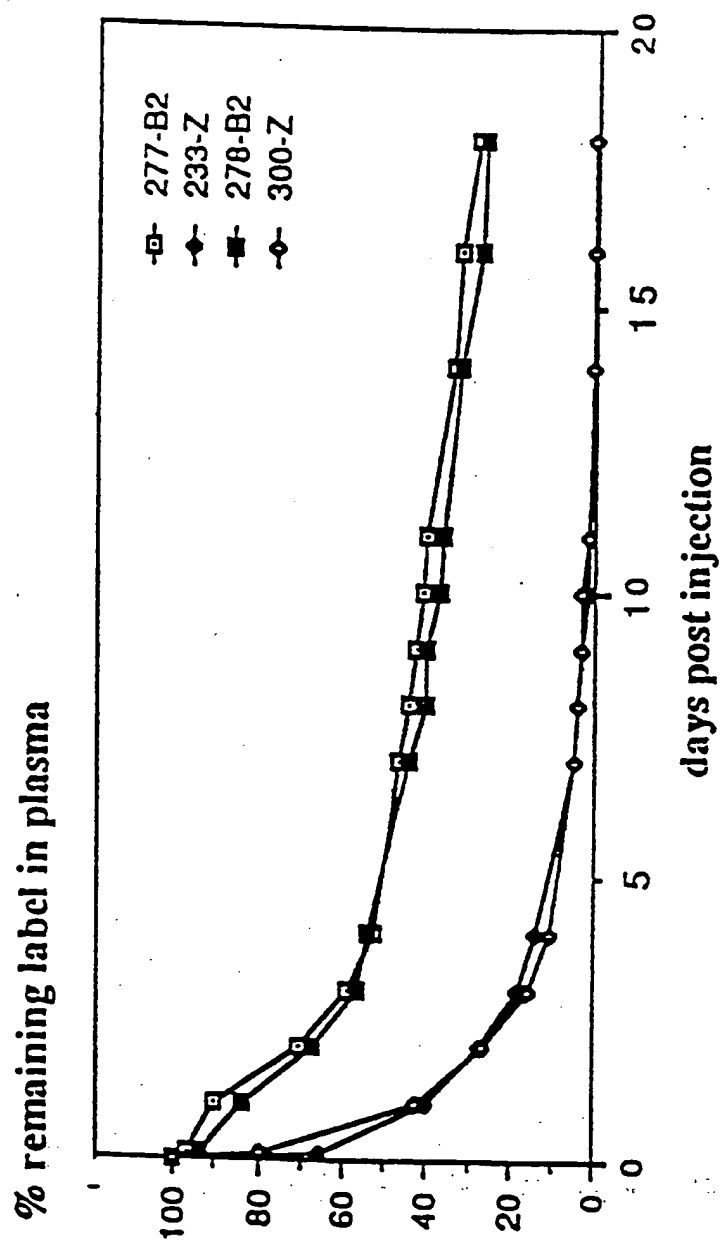
1. A process for extending the half-life in vivo by a biologically active protein or peptide, characterized by covalently coupling said protein or peptide to a polypeptide fragment capable of binding to a serum protein, whereby when administering the resulting protein or peptide conjugate its binding to the serum protein results in extended biological activity.
2. A process according to claim 1, wherein said covalent coupling is performed by recombinant DNA technology.
3. A process according to claim 1, wherein said covalent coupling is performed chemically.
4. A process according to claim 1, 2 or 3, wherein said polypeptide fragment is capable of binding to serum albumin.
5. A process according to claim 4, wherein said serum albumin is of mammal origin.
6. A process according to any preceding claim, wherein said polypeptide fragment originates from staphylococcal protein A or staphylococcal protein G.
7. The use of the protein or peptide conjugate of any preceding claim for manufacturing a medicament which, when administered to a mammal including man, shows extended half-life in vivo.
8. The use according to claim 7, wherein the medicament is an injectable form.
9. The use according to claim 8, wherein the medicament is in the form of an injectable solution or suspension.
10. A method of treating a mammal including man, comprising administering to such mammal a biologically effective amount of a protein or a peptide conjugate as defined herein.

Fig. 1



2/6

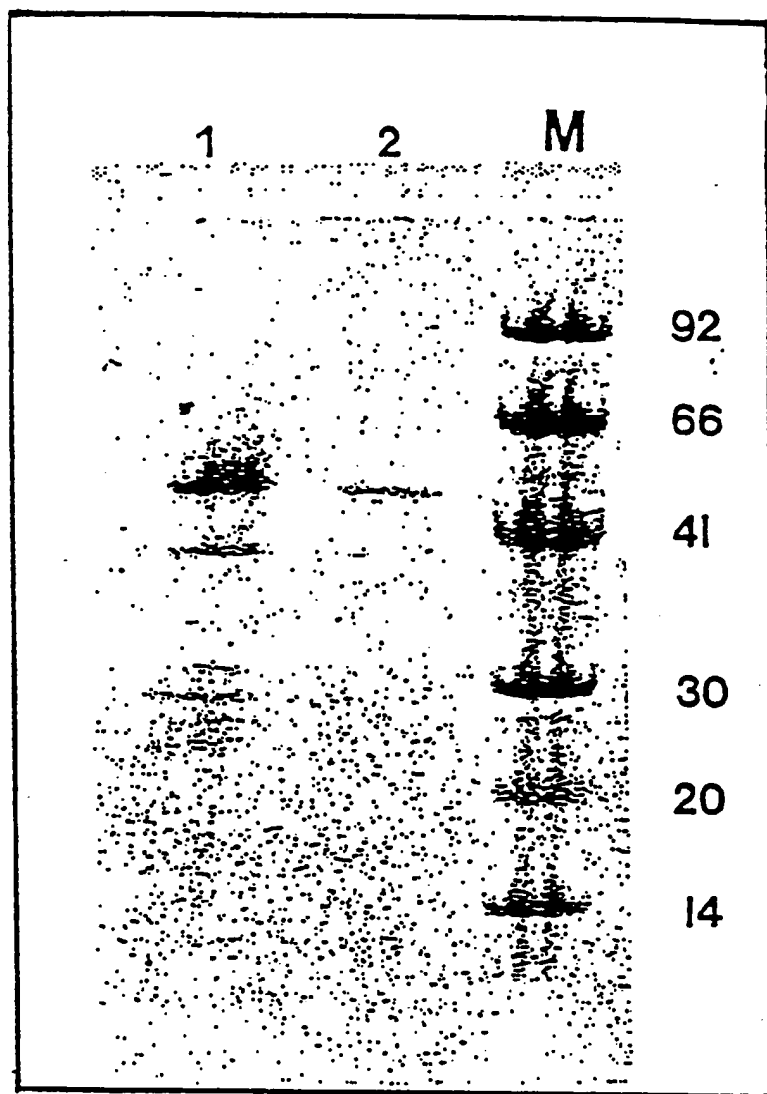
Fig. 2



SUBSTITUTE SHEET

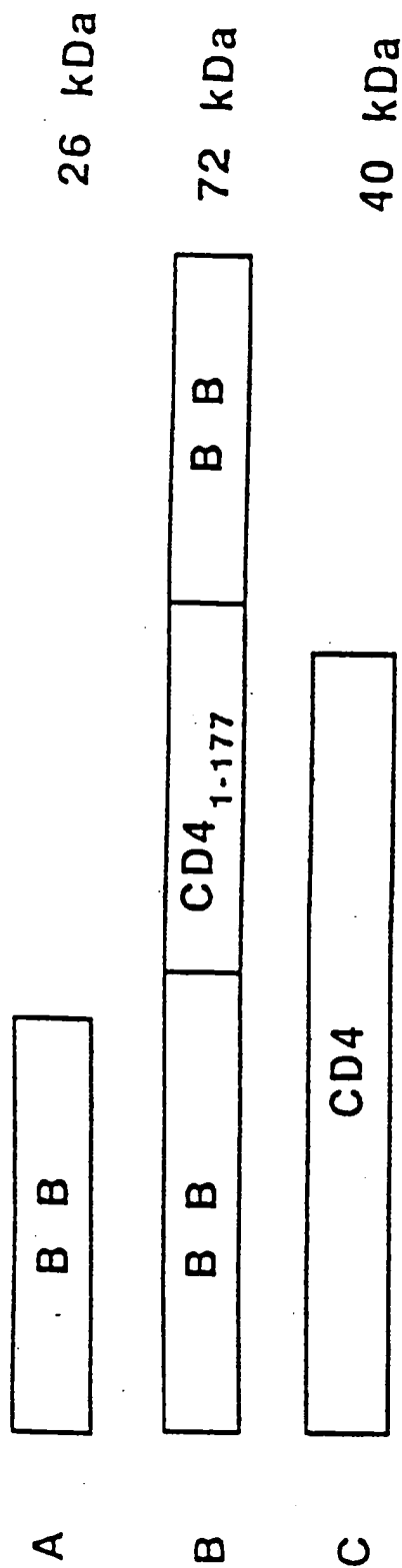
3/6

Fig. 3



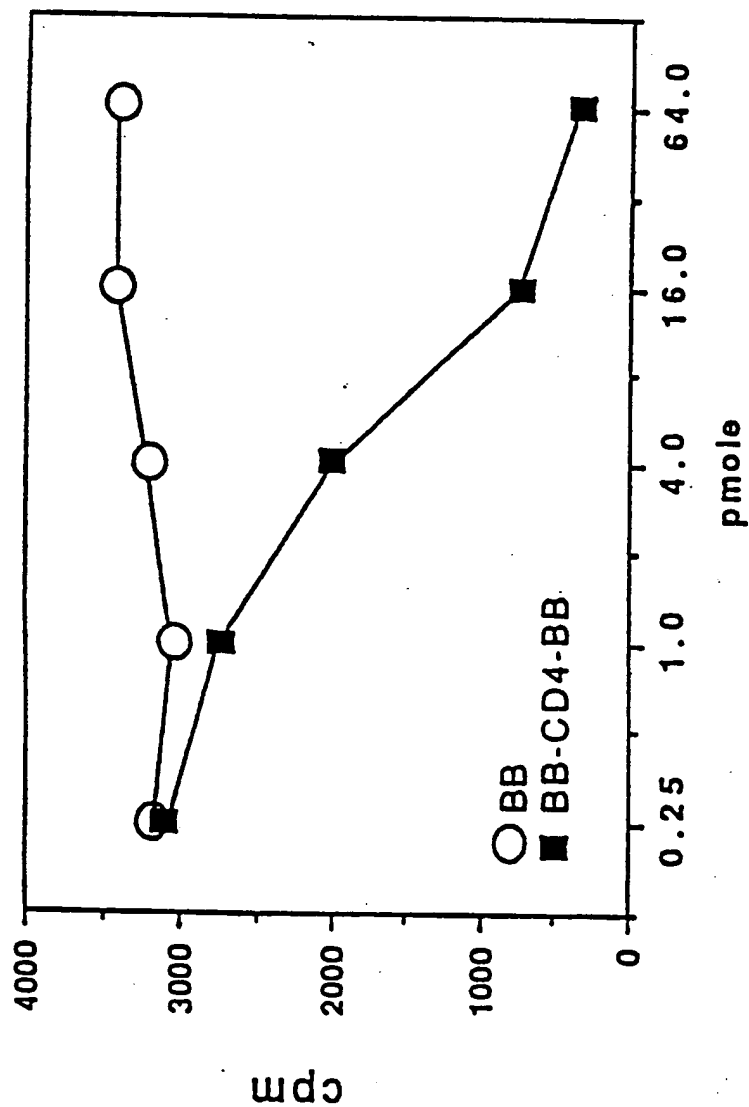
SUBSTITUTE SHEET

Fig. 4

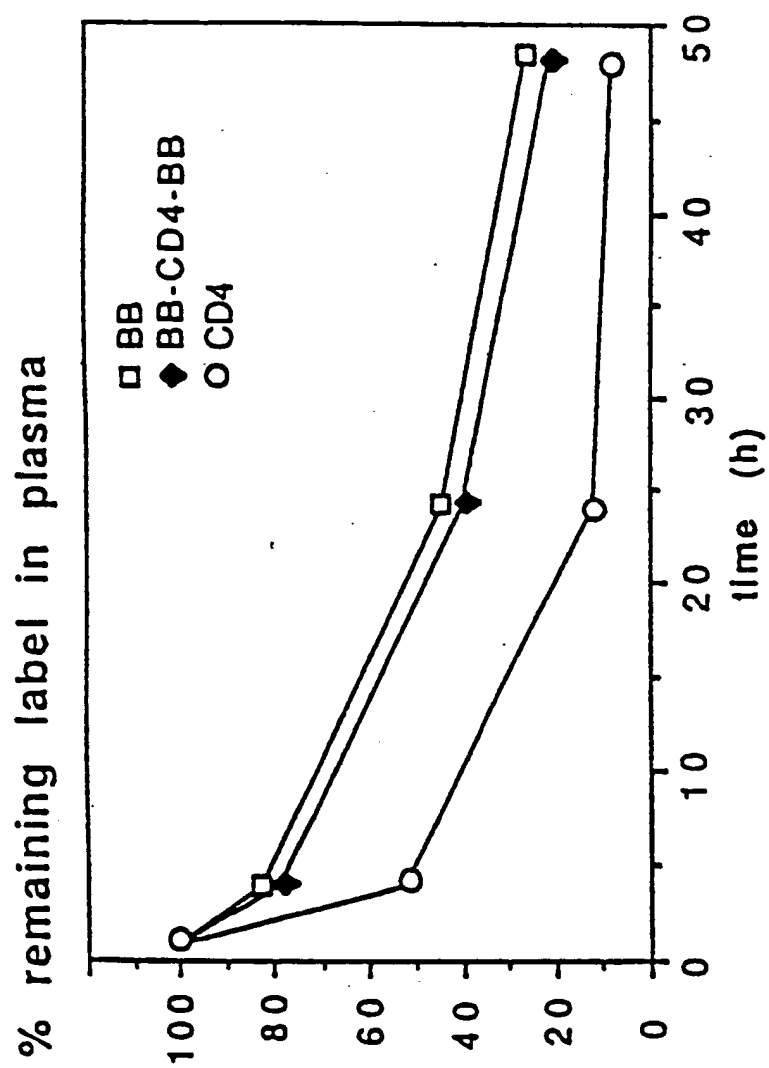


5/6

Remaining label in wells after incubation with
different amounts of inhibitor.



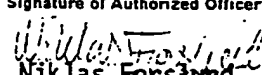
SUBSTITUTE SHEET

Fig. 6

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00509

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: A 61 K 37/02, 47/48, C 07 K 13/00, C 12 N 15/62		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	A 61 K; C 07 K; C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A2, 0136835 (HYBRITECH INCORPORATED) 10 April 1985, see page 2, line 23 - page 3, line 23 and claims --	1,3,5,7- 9
A	EP, A1, 0317641 (SAGAMI CHEMICAL RESEARCHCENTER ET AL) 31 May 1985, see page 3, lines 6-18 and claims --	1-9
A	WO, A1, 8905140 (A BRADWELL) 15 June 1989, see page 6, lines 5-25; abstract and claims --	1-9
A	US, A, 4703039 (J J HAWIGER ET AL) 27 October 1987, see column 2, line 60 - column 3, line 54 --	1-9
<p>[*] Special categories of cited documents: ¹⁰</p> <p>^{"A"} document defining the general state of the art which is not considered to be of particular relevance</p> <p>^{"E"} earlier document but published on or after the international filing date</p> <p>^{"L"} document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>^{"O"} document referring to an oral disclosure, use, exhibition or other means</p> <p>^{"P"} document published prior to the international filing date but later than the priority date claimed</p> <p>^{"T"} later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>^{"X"} document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>^{"Y"} document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>^{"&"} document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
19th October 1990	1990 -10- 24	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Niklas Forslund	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	<p>Proc.Natl.Acad.Sci, Vol. 86, June 1989, B Hammarberg et al: "Dual affinity fusion approach and its use to express recombinant human insulin like growth factor II", pages 4367-4371 see the whole article</p> <p>--</p>	1-9
A	<p>Dialog Information Service, File 155: Medline 66-90/Nov, accession no. 06820435, Medline accessions no. 89122435, T Ogino et al: "Chemical modification of superoxide dismutase. Extension of plasma half life of the enzyme through the reversible binding to the circulating albumin", & Int J Pept Protein Res, Aug 1988, 32(2) p153-9 see abstract</p> <p>--</p> <p>-----</p>	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers...10..., because they relate to subject matter not required to be searched by this Authority, namely:
Method for treating the human or animal body according to PCT rule 39(iv).
2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 90/00509**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **90-09-27**
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0136835	85-04-10	AU-B- 593208	90-02-08
		AU-D- 3393584	85-03-29
		CA-A- 1249222	89-01-24
		GB-A-B- 2148299	85-05-30
		JP-T- 60502104	85-12-05
		WO-A- 85/00974	85-03-14

EP-A1- 0317641	85-05-31	WO-A- 88/09343	88-12-01

WO-A1- 8905140	89-06-15	AU-D- 2825789	89-07-05

US-A- 4703039	87-10-27	AU-D- 4159185	85-11-01
		EP-A- 0180596	86-05-14
		US-A- 4666884	87-05-19
		WO-A- 85/04585	85-10-24

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.